Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Canine Parvovirus in Cell Culture

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Supplemental Assay Method for the Titration of Canine Parvovirus in Cell Culture

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Supplemental Assay Method for the Titration of Canine Parvovirus in Cell Culture

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* test method for assaying modified-live canine parvovirus (CPV) vaccines for viral content. The method uses the Crandall feline kidney (CRFK) cell line as the test system. Presence or absence of CPV is determined by staining inoculated cell cultures by an indirect fluorescent antibody (IFA) technique.

1.2 Keywords

Canine parvovirus; CPV; IFA; potency test; $TCID_{50}$; viral titration; *in vitro*

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] $36^0 \pm 2^0$C, high humidity, 5 ± 1% CO_2 (CO_2 incubator) meeting the requirements of the current version of GDOCSOP004

2.1.2 Incubator,[2] $36^0 \pm 2^0$C, aerobic

2.1.3 Water bath,[3] $36^0 \pm 2^0$C

2.1.4 Microscope,[4] ultraviolet (UV) light

2.1.5 Vortex mixer[5]

2.1.6 Micropipettor,[6] 200 µl with tips[7]

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[1] Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750 or equivalent

[2] Model 2, Precision Scientific, 3737 West Cortland St., Chicago, IL 60647 or equivalent

[3] Cat. No. 66648, Precision Scientific or equivalent

[4] Model BH2, Olympus America, Inc., 2 Corporate Ctr. Dr., Melville, NY 11747 or equivalent

[5] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent

[6] Cat. No. P-200, Rainin Instrument Co., P.O. Box 4026, Mack Rd., Woburn, MA 01801-4628 or equivalent

[7] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent

2.1.7 Microscope slide glass staining dish with rack (glass staining dish)[8]

2.2 Reagents/supplies

2.2.1 CPV Reference,[9] KB5 strain

2.2.2 CRFK cell culture,[10] free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

2.2.3 Feline panleukopenia virus antiserum[11] (FPV Antiserum)

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts without bicarbonate[12]

2.2.4.2 2.2 g sodium bicarbonate ($NaHCO_3$)[13]

2.2.4.3 Dissolve with 900 ml deionized water (DW).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamine[14] to 10 ml DW. Heat to $60^0 \pm 2^0$C until dissolved. Add to **Section 2.2.5.3** with constant mixing.

2.2.4.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[15]

2.2.4.6 Sterilize through a 0.22-µm filter.[16]

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[8] Cat. No. 121, Shandon Lipshaw, 171 Industry Dr., Pittsburg, PA 15275 or equivalent

[9] Seed quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

[10] CCL-94, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776

[11] Reference quantities available upon request from the CVB-L or equivalent

[12] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

[13] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

[14] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

[15] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

[16] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

2.2.4.7 Aseptically add:

1. 25 units/ml penicillin[17]

2. 50 µg/ml gentamicin sulfate[18]

3. 100 µg/ml streptomycin[19]

2.2.4.8 Store at $4^0 \pm 2^0$C.

2.2.5 Growth Medium

2.2.5.1 920 ml of MEM

2.2.5.2 Aseptically add:

1. 70 ml gamma irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine[20]

2.2.6 Dulbecco's phosphate buffered saline (DPBS)

2.2.6.1 8.0 g sodium chloride (NaCl)[21]

2.2.6.2 0.2 g potassium chloride (KCl)[22]

2.2.6.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)[23]

2.2.6.4 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \cdot 6H_2O$)[24]

2.2.6.5 Dissolve reagents with 900 ml DW.

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[17]Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

[18]Cat. No. 0061-0464-04, Schering Laboratories or equivalent

[19]Cat. No. S-9137, Sigma Chemical Co. or equivalent

[20]L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

[21]Cat. No. 3624-01, J.T. Baker, Inc. or equivalent

[22]Cat. No. P217-500, Fisher Scientific Co., 2000 Park Ln., Pittsburg, PA 15275 or equivalent

[23]Cat. No. 3246-01, J.T. Baker, Inc. or equivalent

[24]Cat. No. M33-500, Fisher Scientific Co. or equivalent

2.2.6.6 Dissolve 1.03 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[25] with 10 ml DW, heat to $60^0 \pm 2^0$C until dissolved; add to **Section 2.2.6.5** with constant mixing.

2.2.6.7 Dissolve 0.1 g calcium chloride, anhydrous ($CaCl_2$)[26] with 10 ml DW; add slowly to **Section 2.2.6.6** to avoid precipitation.

2.2.6.8 Q.S. to 1000 ml with DW, adjust pH to 7.0-7.3 with 2N HCl.

2.2.6.9 Sterilize through a 0.22-µm filter.

2.2.7 Glass slides, 8 chamber[27] (Lab-Tek® Slides)

2.2.8 Polystyrene tubes, 12 x 75 mm[28]

2.2.9 Goat anti-cat IgG (H&L) fluorescein isothiocyanate labeled conjugate (Anti-cat Conjugate)[29]

2.2.10 100% Acetone[30]

2.2.11 Tuberculin syringe,[31] 1 ml and needle, 20 ga x 1.5 in[32]

2.2.12 Self-refilling repetitive syringe, 2 ml[33]

2.2.13 Pipette, 10 ml[34]

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[25]Cat. No. 3828-01, J.T. Baker, Inc. or equivalent
[26]Cat. No. 4225-05, J.T. Baker, Inc. or equivalent
[27]Cat. No. 177402, Nunc, Inc., 2000 N. Aurora Rd., Naperville, IL 60563 or equivalent
[28]Falcon® 2058, Becton Dickinson Labware, 1 Becton Dr., Franklin Lakes, NJ 07417 or equivalent
[29]Cat. No. 102-015-003, Jackson ImmunoResearch Laboratories, Inc., 872 W. Baltimore Pike, West Grove, PA 19390 or equivalent
[30]Cat. No. A18-4, Fisher Scientific Co. or equivalent
[31]Cat. No. 309602, Becton Dickinson Labware or equivalent
[32]Cat. No. 250107, Becton Dickinson Labware or equivalent
[33]Wheaton®, Cat. No. 13-689-50C, Fisher Scientific Co. or equivalent
[34] Falcon 7530, Becton Dickinson Labware or equivalent

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation of animal viruses.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of inoculation, set a water bath at $36^0 \pm 2^0$C.

3.2.2 On the day of the IFA test, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DW.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK Slides

3.3.1.1 Cells are prepared from healthy, confluent CRFK cells, that are maintained by passing every 3 to 4 days. On the day of test initiation, add 0.4 ml/chamber of $10^{5.2}$ to $10^{5.4}$ cells/ml diluted in Growth Media into all chambers of a Lab-Tek® slide. Prepare sufficient Lab-Tek® Slides to allow 25 wells for controls and 20 wells for each Test Serial. Incubate at $36^0 \pm 2^0$C in a CO_2 incubator. These become the CRFK Slides.

3.3.1.2 Use seeded CRFK Slides within 4 hr.

3.3.2 Preparation of the CPV Working Reference

3.3.2.1 On the day of inoculation, rapidly thaw a vial of CPV Reference in a $36^0 \pm 2^0$C water bath.

3.3.2.2 With the self-refilling repetitive syringe, dispense 1.8 ml of MEM into each of 7, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-7}.

3.3.2.3 With a micropipettor, transfer 200 µl of the CPV Reference to the tube labeled 10^{-1}; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 200 µl from the 10^{-1} labeled tube **(Section 3.3.2.3)** to the 10^{-2} tube; mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each subsequent dilution, transferring 200 µl from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.3.3 Preparation of Working FPV Antiserum

On the day of the IFA test, dilute FPV Antiserum in DPBS to the IFA working dilution on the CVB-L Reference and Reagent Sheet or as determined for that specific antiserum.

3.3.4 Preparation of Working Anti-cat Conjugate

On the day of the IFA test, dilute Anti-cat Conjugate in DPBS to the working dilution according to the manufacturer's recommendations.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). On the day of inoculation, rehydrate a vial of the Test Serial by transferring 1.0 ml for a 1-ml-dose vaccine, 0.5 ml for 1/2-ml-dose vaccines, etc., of the provided diluent into the vial containing the lyophilized Test Serial. Use a sterile 1.0-ml syringe and an 18-ga x 1.5-in needle; mix by vortexing. Incubate for 15 ± 5 min at room temperature (RT) (23^0 ± 2^0C).

3.4.2 Using the self-refilling repetitive syringe, dispense 1.8 ml MEM into each of 6, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-6}.

3.4.3 With a micropipettor, transfer 200 µl from the rehydrated Test Serial vial to the tube labeled 10^{-1}; mix by vortexing.

3.4.4 Using a new pipette tip, transfer 200 µl from the 10^{-1} labeled tube **(Section 3.4.3)** to the 10^{-2} tube; mix by vortexing.

3.4.5 Repeat **Section 3.4.4** for each subsequent dilution, transferring 200 µl from the previous dilution to the next dilution tube, until the tenfold dilution series is completed.

4. Performance of the test

4.1 Inoculate 5 chambers/dilution of a CRFK Slide with 100 µl/chamber from dilutions 10^{-6} through 10^{-3} of the Test Serial. Tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-6} through 10^{-3}).

4.2 Inoculate 5 chambers/dilution of a CRFK Slide with 100 µl/chamber, from dilutions 10^{-7} through 10^{-4} of the CPV Working Reference.

4.3 Five uninoculated chambers serve as a Negative Cell Control.

4.4 Incubate CRFK Slides in a $36^0 \pm 2^0$C CO_2 incubator for 120 ± 12 hr.

4.5 Following incubation, decant the media from the CRFK Slide and remove the plastic walls by twisting them away from the CRFK Slide, leaving the gasket attached to the slide.

4.6 Place the CRFK Slides in a slide rack; place the rack in a glass staining dish filled with DPBS. Incubate for 15 ± 5 min at RT.

4.7 Discard the DPBS; fix the CRFK Slides in 100% Acetone for 15 ± 5 min at RT. Remove and allow to air dry.

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4.8 Pipette 75 ± 25 µl of the Working FPV Antiserum into each chamber of the CRFK Slides. Incubate for 30 ± 5 min in the aerobic incubator at 36^0 ± 2^0C.

4.9 Wash per **Section 4.6.**

4.10 Pipette 75 ± 25 µl of the Working Anti-cat Conjugate into each chamber of the CRFK Slides. Incubate for 30 ± 5 min in the aerobic incubator at 36^0 ± 2^0C.

4.11 Wash per **Section 4.6.**

4.12 Rinse the CRFK Slides with DW; allow to air dry.

4.13 Read at 100-200X magnification with a UV-light microscope; examine the cell monolayer for typical CPV apple-green nuclear fluorescence.

4.13.1 Chambers containing 1 or more cells with specific CPV fluorescence are considered to be positive.

4.13.2 Results are recorded as number of CPV positive chambers versus total number of chambers examined for each dilution of a Test Serial and the CPV Working Reference.

4.14 Calculate the 50% tissue culture infective dose ($TCID_{50}$) of the Test Serial and the CPV Working Reference using the method of Spearman-Kärber as modified by Finney.

Example:
10^{-3} dilution of Test Serial = 5/5 chambers IFA positive
10^{-4} dilution of Test Serial = 5/5 chambers IFA positive
10^{-5} dilution of Test Serial = 2/5 chambers IFA positive
10^{-6} dilution of Test Serial = 0/5 chambers IFA positive

Spearman-Kärber calculation of total IFA positive wells (12), using 5 wells per dilution = 1.9 log

Log_{10} of reciprocal dilution (10^{-3}) = 3.0 log

Log_{10} of reciprocal of dose factor:

$$\frac{0.1 \text{ ml inoculum}}{1 \text{ ml dose}} = \frac{1}{10} \quad = 1.0 \log$$

Total = 5.9 log

Titer of the Test Serial is $10^{5.9}$ $TCID_{50}$.

5. Interpretation of the test results

5.1 Validity requirements:

5.1.1 The calculated $TCID_{50}$ titer of the CPV Working Reference must fall within ± 2 standard deviations (SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The lowest inoculated dilution of the CPV Working Reference must exhibit a 100% positive IFA reaction (5/5), and the highest (most dilute) must exhibit a negative IFA reaction (0/5).

5.1.3 The Negative Cell Control must not exhibit any cytopathic effect, specific CPV fluorescence, or cloudy media that would indicate contamination.

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

5.3 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Serial is considered **SATISFACTORY**.

5.4 If the validity requirements are met, but the titer of the Test Serial is less than the required minimum titer contained in the APHIS filed Outline of Production for the product under test, the Test Serial may be retested in accordance with the 9 CFR, Part 113.8.

6. Report of test results

Report results as the $TCID_{50}$ per dose of the Test Serial.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.317, U.S. Government Printing Office, 1999.

7.2 Cottral, GE, (Ed.), 1978. *Manual of standardized methods for veterinary microbiology.* Comstock Publishing Associates, Ithaca, NY. pg. 731.

7.3 Finney, DJ, 1978. *Statistical methods in biological assay.* Griffin, London. 3rd edition, pg. 508.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.